UltraRapid Communication

Regulation of the Growth Arrest and DNA Damage-Inducible Gene 45 (GADD45) by Peroxisome Proliferator-Activated Receptor γ in Vascular Smooth Muscle Cells

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Abstract—Peroxisome proliferator-activated receptor (PPAR) γ is activated by thiazolidinediones (TZDs), widely used as insulin-sensitizing agents for the treatment of type 2 diabetes. TZDs have been shown to induce apoptosis in a variety of mammalian cells. In vascular smooth muscle cells (VSMCs), proliferation and apoptosis may be competing processes during the formation of restenotic and atherosclerotic lesions. The precise molecular mechanisms by which TZDs induce apoptosis in VSMCs, however, remain unclear. In the present study, we demonstrate that the TZDs rosiglitazone (RSG), troglitazone (TRO), and a novel non-TZD partial PPARγ agonist (nTZDpa) induce caspase-mediated apoptosis of human coronary VSMCs. Induction of VSMC apoptosis correlated closely with an upregulation of growth arrest and DNA damage-inducible gene 45 (GADD45) mRNA expression and transcription, a well-recognized modulator of cell cycle arrest and apoptosis. Using adenoviral-mediated overexpression of a constitutively active PPARγ mutant and the irreversible PPARγ antagonist GW9662, we provide evidence that PPARγ ligands induce caspase-mediated apoptosis and GADD45 expression through a receptor-dependent pathway. Deletion analysis of the GADD45 promoter revealed that a 153-bp region between −234 and −81 bp proximal to the transcription start site, containing an Oct-1 element, was crucial for the PPARγ ligand-mediated induction of the GADD45 promoter. PPARγ activation induced Oct-1 protein expression and DNA binding and stimulated activity of a reporter plasmid driven by multiple Oct-1 elements. These findings suggest that activation of PPARγ can lead to apoptosis and growth arrest in VSMCs, at least in part, by inducing Oct-1–mediated transcription of GADD45. The full text of this article is available online at http://www.circresaha.org. (Circ Res. 2003;93:e38-e47.)

Key Words: peroxisome proliferator-activated receptor γ ■ apoptosis ■ vascular smooth muscle ■ octamer transcription factor

The pathogenesis of atherosclerosis and postangioplasty restenosis involves an excessive proliferation of vascular smooth muscle cells (VSMCs).1–3 Although many studies have focused on alterations in the regulation of cell growth as a fundamental feature during atherogenesis and neointimal hyperplasia after percutaneous coronary revascularization, it is becoming increasingly evident that perturbations in the regulation of cell death may be of equal importance.4

Peroxisome proliferator-activated receptor (PPAR) γ is a transcription factor belonging to the nuclear hormone receptor gene super-family.5 PPARγ is expressed in VSMCs and prominently upregulated in response to mechanical injury of the arterial wall.6,7 PPARγ heterodimerizes with the retinoid X receptor (RXR) and binds to specific response elements termed peroxisome proliferator–response elements (PPRE).8 PPARγ functions as a ligand-dependent transcription factor, which on ligand binding undergoes a conformational change disrupting a corepressor complex and leading to coactivator recruitment resulting in the transcriptional activation of target genes.9,10

Thiazolidinediones (TZD) are synthetic ligands for PPARγ and are commonly used as insulin-sensitizing agents in the treatment of type 2 diabetes.11 Pharmacological activation of PPARγ by TZD and non-TZD PPARγ ligands inhibits VSMC proliferation at the G1→S phase transition of the cell cycle by suppressing mitogen-induced phosphorylation of the
retinoblastoma protein, which is required for E2F release and expression of minichromosome maintenance proteins that are essential regulators of the DNA replication.12,13 This inhibition of VSMC proliferation by PPARγ ligands correlates with a potent suppression of neointima formation after balloon-injury in rat models of insulin resistance, as well as in animals with normal insulin sensitivity in vivo.14-17 In addition, recent studies have shown that TZDs not only inhibit cell growth, but they can also induce apoptosis in diverse cell types; including endothelial cells,18 VSMCs,19,20 monocyte-derived macrophages,21 and various human cancer cells.22-25

The growth arrest and DNA damage-inducible (GADD) gene GADD45 is a member of a group of genes induced by agents that damage DNA and/or cause growth arrest.26 Increased GADD45 gene expression has been detected in many mammalian cell types and has been implicated in terminal differentiation,27 growth suppression,28,29 and apoptosis.30,31 Although the exact function of GADD45 remains unclear, evidence has emerged that GADD45 is a cell cycle–regulated nuclear protein that reaches maximal levels in the G1 phase of the cycle.32 Through its association with Cdc2, GADD45 disrupts the interactions of Cdc2 with cyclin B1 and, thus, may induce G1/M arrest.33 The GADD genes, therefore, may represent a unique target for drugs that induce cell cycle arrest, apoptosis, and differentiation such as PPARγ ligands.

The molecular mechanisms by which TZDs induce apoptosis in VSMCs and whether they involve a PPARγ-dependent pathway remain unclear. In the present study, we demonstrate that PPARγ ligands induce caspase-mediated apoptosis of human coronary VSMCs. The ability of these PPARγ ligands to induce VSMC apoptosis correlated with an induction of GADD45 expression at the transcriptional level. By using adenoviral-mediated overexpression of a constitutively active form of PPARγ, we provide evidence that the mechanism of action by which TZDs and non-TZDs induce apoptosis and GADD45 transcription occurs through a PPARγ-dependent pathway. The PPARγ ligand-responsive element in the GADD45 promoter was mapped by promoter-deletion studies to a 153-bp region between −234 and −81 bp relative to the transcription start site that harbors an Oct-1 motif. PPARγ activation induced Oct-1 protein expression and increased its DNA binding and transcriptional activity. These results, therefore, identify the attenuation of Oct-1–mediated induction of GADD45 by PPARγ as a novel mechanism for regulating VSMC growth and survival.

Materials and Methods

Cell Culture

Primary human coronary artery vascular smooth muscle cells (HCSCMs, Clonetics coronary artery smooth muscle cell systems) were commercially obtained from Cambrex Bio Science Walkersville, Inc (East Rutherford, NJ). Early passage (fourth to ninth) cells were grown to 70% to 80% confluence in SmBM-2 medium containing 5% FBS, 2 ng/mL human basic fibroblast growth factor, 0.5 ng/mL human epidermal growth factor, 50 μg/mL gentamycin, and 5 μg/mL bovine insulin. Growing cells were treated with each compound for the indicated time. Troglitazone (TRO) was kindly provided by Parke-Davis and rosiglitazone by Smith Kline Beecham. The compound nTZDpα (1-(p-chlorobenzyl)-3-chloro-3-phenylthio benzyl-2-yl carboxylic acid) was kindly provided by Dr David E. Moller (Merck Research Laboratories, Rahway, NJ), and the irreversible PPARγ antagonist GW9662 was a kind gift from Dr Timothy M. Willson (Glaxo Smith Kline, Research Triangle Park, NC). For all data shown, individual experiments were performed from separate lots of HCSMCs.

Annexin V Flow Cytometry

HCSMCs were treated with the PPARγ ligands for 48 hours or pretreated with 20 μmol/L of the pan-caspase inhibitor Z-VAD-FMK (R&D Systems) for 3 hours before stimulation with 30 μmol/L of the PPARγ ligands or vehicle (DMSO). All experiments were performed with exponentially growing cells in SmBM-2 containing growth factors and 5% FBS. Annexin V flow cytometry was performed using a commercially available annexin V fluorescein isothiocyanate (FITC) assay (Oncogene Research Products). Briefly, cells were washed with PBS and trypsinized with 0.125% trypsin until cells appeared to detach by microscopic evaluation. Cells were then released by firm tapping, resuspended in media to 1×10⁶ cells/mL, and incubated for 30 minutes with the annexin V-FITC–conjugated antibody and stained with propidium iodide. Annexin V–FITC binding was analyzed by flow cytometry collecting the fluorescence of 10,000 cells using a FACScan (Becton Dickinson) according to the manufacturer’s instructions. The total number of apoptotic cells was expressed as fold induction of annexin V–FITC–positive cells compared with vehicle (DMSO)-treated cells.

Western Immunoblotting

Cells were harvested at the indicated time after treatment with the PPARγ ligand and sonicated in solubilization buffer (20 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 1 mmol/L EDTA; 1 mmol/L EGTA, 1% Triton X-100; 2.5 mmol/L sodium pyrophosphate; 1 mmol/L sodium vanadate; 10 μg/mL each aprotinin and leupeptin; 1 mmol/L phenylmethylsulfonyl fluoride). Whole cell lysates were cleared by centrifugation and protein concentrations were determined by Lowry assay. Nuclear extracts were isolated using the NuCLEAR extraction kit according to the manufacturers instructions (Sigma). Cell lysates containing equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis. Protein was transferred to a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech). After blocking in 20 mmol/L Tris-HCl (pH 7.6) containing 150 mmol/L NaCl, 0.1% Tween-20, and 5% (wt/vol) non-fat dry milk, blots were incubated with specific antibodies for caspase-3 (Cell Signaling Technology), PPARγ, or Oct-1 (Santa Cruz Biotechnology) and cohybridized with β-actin (Santa Cruz Biotechnology) to monitor equivalent loading in different lanes. Immunoreactive bands were visualized by incubation with peroxidase-conjugated anti-mouse IgG antibody (Amersham Pharmacia Biotech). The antigen-antibody complexes were detected using ECL (Amersham Pharmacia Biotech). Quantification of the Western blots was performed by densitometry.

Isolation of RNA and Northern Blotting

Total RNA was isolated using Trizol reagent (Life Technologies) as described by the manufacturer. Fifteen micrograms of total RNA were denatured in formamide/formaldehyde and electrophoresed through 1% formaldehyde-containing agarose gels. After electrophoresis, RNA was transferred to nylon membrane (Hybond N+, Amersham Pharmacia Biotech) by capillary blotting and fixed by UV cross-linking. Hybridization was performed using PerfectHyb Plus hybridization buffer (Sigma) as directed. cDNA for GADD45 was obtained from ATCC (No. 1150356, ATCC). The cDNAs used in the hybridization were radiolabeled with [α-32P] dCTP (ICN) using Rediprime II random prime labeling system (Amersham Pharmacia Biotech). Blots were cohybridized with CDNA encoding the constitutively expressed housekeeping gene Chinese hamster ovary gene B (CHOB) to assess equal loading of samples.
Adenoviral Infection of HCSMCs
To generate constitutively active PPARγ the VP16 transactivation domain of the herpes simplex virus (HSV) was fused to the N-terminus of PPARγ as previously described. Recombinant type 5 adenovirus overexpressing this constitutively active PPARγ mutant was generated using the Adeno X Expression System (Clontech Inc) and designated as Adx-CA-PPARγ. Adenovirus overexpressing only the VP16 transactivation domain (pTet/VP16, obtained from Clontech Inc) or green fluorescent protein (GFP) were generated similarly and used as control (Adx-GFP, Adx-VP16) in all experiments. HCSMCs were infected with 1, 30, or 100 plaque forming units (PFU)/cell adenovirus in SmiBM-2 containing 5% FBS for 48 hours.

RNA Stability Assay
HCSMCs grown for 48 hours in the presence of 30 μmol/L TRO or vehicle (DMSO) were treated with actinomycin D (5 μg/mL), and at different time intervals, cells were collected and RNA was isolated. Total RNA expression was examined by Northern blot analysis using radiolabeled probes for GADD45 or CHOB. Hybridization signals were quantified by densitometry, normalized to CHOB expression, and mRNA half-life ($T_1/2$) was calculated using the equation $C = Ce^{-kt}$, where $C$ is the remaining mRNA level at time point $t$, $Ce$ is the amount of RNA at zero time of actinomycin D addition, and $k_e$ is the mRNA decay constant.

Plasmids and Transient Transfection
The GADD45 luciferase reporter constructs and the construction of the IL-8OCx6, IL-8OmCx6, and IL-8mOCx6 have been previously described. One microgram DNA was transfected using LipofectAMINE 2000 (Invitrogen). Twenty-four hours after transfection, cells were treated with the indicated PPARγ ligand. Luciferase activity was assayed using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Transfection efficiency was adjusted by normalizing firefly luciferase activities to an internal control of Renilla luciferase.

Electrophoretic Mobility Shift Assay
For electrophoretic mobility shift assays (EMSA), a radioactive Oct-1 consensus oligonucleotide (5′-GTGCAGATGCAAATCACTAGAA-3′, Promega), labeled with T4 polynucleotide kinase and [γ-32P]ATP (ICN) was incubated with 4 μg of nuclear extract for 20 minutes in gel shift binding buffer (5 mmol/L MgCl2, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris [pH 7.5], and 0.25 μg/μL poly dIdC in 20% glycerol) and analyzed by electrophoresis using a 4% nondenaturing acrylamide gel in 0.5× TBE buffer. Gels were dried, and autoradiography was performed. For competitive and noncompetitive oligonucleotide assays 100-fold labeled double-stranded Sp-1 (Promega) or unlabeled double-stranded Oct-1 were added to the nuclear extracts 20 minutes before the addition of the radiolabeled probe. For supershift experiments, 4 μg of nuclear extract were incubated with 0.5 μg of an Oct-1 antibody (Santa Cruz Biotechnology) for 20 minutes after incubation of the radiolabeled probe with nuclear proteins.

Statistics
Data were expressed as mean±SEM. Statistical significance was determined using the unpaired Student’s $t$ test or ANOVA when comparing multiple groups. Values of $P<0.05$ were considered as statistically significant.

Results
PPARγ Ligands Induce Apoptosis of Human Coronary VSMCs
To determine whether PPARγ activation could induce VSMC apoptosis, human coronary VSMCs were treated with various

![Figure 1. PPARγ ligands induce apoptosis in vascular smooth muscle cells. A, Human coronary VSMCs were cultured in the presence of the indicated PPARγ ligand (1, 10, and 30 μmol/L) for 48 hours or pretreated with the pan-caspase inhibitor Z-VAD-FMK (Z-VAD, 20 μmol/L) 3 hours before stimulation with 30 μmol/L of the indicated PPARγ ligand. Apoptosis was analyzed by annexin V-FITC flow cytometry and expressed as the fold induction of the vehicle (DMSO). Data are presented as mean±SEM of 3 independently performed experiments (*$P<0.05$ vs vehicle; †$P<0.05$ vs stimulation with 30 μmol/L of the indicated PPARγ ligands alone). B, Human coronary VSMCs were stimulated with 30 μmol/L of indicated PPARγ ligand. Forty-eight hours after stimulation, caspase-3 protein expression was measured by Western immunoblotting using a monoclonal caspase-3 antibody. To assess loading variability, immunoblots were cohybridized with a specific antibody for β-actin. Autoradiogram shown is representative of 3 independently performed experiments.](image-url)
and to identify the pathway by which activation of PPARγ induces apoptosis, HCSMCs were pretreated with the pan-caspase inhibitor Z-VAD-FMK that potently inhibits caspase enzymes as key executioners of apoptosis. Pretreatment of cells with 20 μmol/L Z-VAD-FMK 3 hours before stimulation with 30 μmol/L of the PPARγ ligand completely prevented RSG, TRO, and nTZDpa-induced apoptosis of HCSMCs. In addition, Western blot analysis (Figure 1B) revealed increased levels of the enzymatically active 17- and 12-kDa proteolytic cleavage products of the inactive 32-kDa caspase-3 precursor in HCSMCs treated for 48 hours with either RSG, TRO, or nTZDpa. In concert, these data indicate that PPARγ ligands induce apoptosis of HCSMCs through a mechanism involving activation of caspases.

GADD45 Expression Is Induced by PPARγ Ligands
GADD45 is prominently induced in growth-arrested cells\(^{28,29}\) and in those undergoing apoptosis.\(^{30,31}\) Because PPARγ activation has been shown to inhibit cell proliferation and/or to induce apoptosis in a wide variety of mammalian cells, we hypothesized that upregulation of GADD45 by PPARγ ligands may be involved in their proapoptotic and antiproliferative activities. Northern blot analysis for GADD45 expression was performed on RNA extracted from HCSMCs treated with TRO for various time-points. Baseline levels of GADD45 mRNA in growing HCSMCs were low, but increased substantially after treatment with TRO (30 μmol/L), reaching maximal levels after 48 hours (Figure 2A). To determine dose-dependent effects of other PPARγ ligands on GADD45 mRNA expression, HCSMCs were cultured in the presence of 1 to 30 μmol/L RSG, TRO, and nTZDpa for 48 hours. Data presented in Figure 2B demonstrate substantial dose-dependent induction of GADD45 mRNA expression after treatment with RSG, TRO, and nTZDpa (RSG 3.2±0.41-, TRO 4.9±0.75-, nTZDpa 3.4±0.53-fold increase at 30 μmol/L, n=3; P<0.05). These data suggest that ligand-dependent activation of PPARγ can lead to growth arrest and induction of apoptosis in vascular smooth muscle cells, at least in part, by inducing GADD45 mRNA expression.

Constitutively Active PPARγ Induces Caspase-Mediated Apoptosis and GADD45 mRNA in HCSMCs
The precise mechanism by which PPARγ ligands exert their antiproliferative and proapoptotic effects and whether it involves PPARγ mediated transactivation of target genes is not known. To determine whether activation of PPARγ induces apoptosis and GADD45 expression, we used a constitutively active form of PPARγ by fusing the herpes virus VP16 transactivation domain to the wild-type PPARγ cDNA.\(^{36}\) This constitutively active PPARγ was subcloned into recombinant adenovirus to permit ubiquitous expression of this engineered nuclear receptor. As shown in Figure 3, we observed faint expression of endogenous PPARγ (≈55 kDa) in whole-cell extracts of uninfected HCSMCs or cells infected with control Adx-VP16 or Adx-GFP. This finding is consistent with our previous observation that expression of endogenous PPARγ protein in VSMCs was detectable only in nuclear fractions, not in whole-cell lysates.\(^{14}\) Infection of HCSMCs with Adx-CA-PPARγ resulted in marked dose-dependent overexpression of constitutively active PPARγ protein, which migrated slower than endogenous PPARγ at ≈70 kDa due to the additional VP16 transactivation domain engrailed at the N-terminus. Similarly, immunoblotting for VP16 revealed fast-migrating dose-dependent expression of VP16 protein in HCSMCs infected with Adx-VP16 and slower migrating (due to the additional PPARγ) VP16 in cells that were infected with Adx-CA-PPARγ. Infection of HCSMCs with the control Adx-GFP or Adx-VP16 had no effect on annexin V binding, caspase-3 cleavage products, or GADD45 mRNA expression. In contrast, annexin V binding
activation have been shown to be regulated by both transcriptional and posttranscriptional mRNA stabilization.37–39 and posttranscriptional mRNA stabilization in HCSMCs and GADD45 mRNA expression and provides evidence that the induction of GADD45 by PPARγ ligands is mediated through a PPARγ-dependent mechanism.

PPARγ Ligands Induce GADD45 Transcription

The PPARγ-mediated induction of GADD45 mRNA could result from either an effect to increase transcription and/or promote enhanced mRNA stability. GADD45 mRNA levels have been shown to be regulated by both transcriptional activation37–39 and posttranscriptional mRNA stabilization depending on the cell type and specific inducer. We next investigated whether treatment with TRO affects stabilization of GADD45 mRNA. HCSMCs were treated for 48 hours with either DMSO or TRO (30 μmol/L) before actinomycin D was added at a final concentration of 5 μg/mL to suppress further transcription during the actinomycin D chase. As shown in Figure 4, treatment with TRO substantially induced GADD45 mRNA expression after 48 hours, whereas the vehicle (DMSO) had no effect on GADD45 expression. GADD45 mRNA levels in both vehicle and TRO-treated cells rapidly decreased within 2 hours. GADD45 mRNA levels were normalized to those of the house-keeping gene CHOB and the mRNA half-lives were calculated. The half-life of GADD45 mRNA in control and TRO-treated cells was calculated as 62 minutes and 72 minutes, respectively. These results demonstrate that PPARγ ligands do not enhance GADD45 mRNA stability in HCSMCs.

To examine the effect of PPARγ ligands on GADD45 transcription, HCSMCs were transiently transfected with a human GADD45 promoter construct containing 2543 bp of 5′-flanking DNA for the GADD45 gene inserted upstream of a luciferase reporter (pG45-Luc).37 Twenty-four hours after transfection, cells were treated with either RSG, TRO, or nTZDpa at 30 μmol/L for an additional 48 hours (Figure 5, top). PPARγ ligands exhibited a negligible effect on HCSMCs transfected with the control empty pGBV2 vector. RSG, TRO, and nTZDpa induced GADD45 promoter activity by 2.96 ± 0.33-, 3.9 ± 0.47-, and 3.21 ± 0.38-fold (n = 3, P < 0.05), respectively. To further elucidate whether PPARγ ligands stimulate GADD45 transcription through a receptor-dependent mechanism, HCSMCs were transfected with the GADD45 promoter construct pG45 and pretreated with the irreversible PPARγ antagonist GW9662 for 3 hours. When

Figure 4. PPARγ ligands have no effect on GADD45 mRNA stability in VSMCs. Human coronary VSMCs were grown for 48 hours in the presence of vehicle (DMSO, left) or 30 μmol/L TRO (right) and treated with actinomycin D (5 μg/mL). At different time intervals, cells were collected and RNA was isolated. Total RNA expression was examined by Northern blot analysis using radiolabeled probes for GADD45 or CHOB. Hybridization signals were quantified by densitometry and GADD45 mRNA expression was normalized to CHOB expression. Quotient from DMSO- or TRO-treated cells at time 0 was arbitrarily defined as 1.
Figure 5. PPARγ ligands induce transcriptional activation of the GADD45 promoter through a receptor-dependent pathway. Human coronary VSMCs were transiently transfected with 1 μg pG45-Luc or pGBV2. Transfected cells were stimulated with vehicle or the indicated PPARγ ligand or were pretreated with the irreversible PPARγ antagonist GW9662 (10 μmol/L) 3 hours before stimulation with the indicated PPARγ ligands. Forty-eight hours after stimulation, luciferase activity was assayed. Transfection efficiency was adjusted by normalizing firefly luciferase activities to renilla luciferase activities generated by cotransfection with 10 ng pRL-CMV. All experiments were repeated at least 3 times. Data are expressed as fold induction over vehicle and presented as mean±SEM (*P<0.05 vs vehicle; #P<0.05 vs stimulation with PPARγ ligands).

endogenous wild-type PPARγ function was pharmacologically blocked by GW9662, RSG, TRO, and nTZDpa failed to activate the GADD45 promoter (Figure 5, bottom). In combination, these results demonstrate that the induction of the GADD45 promoter activity by PPARγ ligands requires functional endogenous PPARγ and occurs through a receptor-dependent mechanism.

Deletion Analysis of the GADD45 Promoter
To identify the PPARγ-responsive elements in the GADD45 promoter, a deletion series of luciferase reporter constructs spanning the different regions of the human GADD45 promoter was used. After transfection of these reporter constructs into HCSMCs, cells were administered with the PPARγ ligands RSG, TRO, or nTZDpa at 30 μmol/L. As shown in Figure 6, the full-length GADD45 promoter was strongly induced by all three PPARγ ligands. Progressive 5’-deletions of the promoter that extended to −81 relative to the transcription start site exhibited almost no induction after treatment with PPARγ ligands. The region between −234 and −81, therefore, contains critical elements required for activation of the GADD45 promoter by PPARγ ligands. Sequence analysis indicates that these regions of the GADD45 promoter harbors an Oct-1 consensus sequence and one CCAAT motif, both of which are known to be crucial for regulating GADD45 expression.17–19

Octamer-Binding Factor Oct-1 Is Essential for the PPARγ Ligand-Mediated Induction of GADD45
To determine whether the Oct-1 and CCAAT motifs play a role in the activation of the GADD45 promoter by PPARγ ligands, we used Oct-1 and CCAAT reporter plasmids, where a hexamer of a 20-bp region containing both Oct-1 and CCAAT consensus sequences was linked to a minimal IL-8 promoter construct and luciferase reporter. In Figure 7, IL-8-OCx6 was transiently transfected into HCSMCs and

Figure 6. Localization of the PPAR-responsive element by 5’-deletion analysis of the GADD45 promoter. Human coronary VSMCs were transiently transfected with various deletion mutants of the human GADD45 promoter. Transfected cells were stimulated with PPARγ ligands as indicated for 48 hours. After stimulation, luciferase activity was assayed. Transfection efficiency was adjusted by normalizing firefly luciferase activities to renilla luciferase activities generated by cotransfection with 10 ng pRL-CMV. All experiments were repeated at least 3 times. Data are expressed as fold induction over vehicle and presented as mean±SEM.

ligands. Schematic representation of the tandem-repeat constructs of the minimal-responsive element and the mutants (left). O indicates Oct-1 consensus sequence; C, CCAAT box. Motifs containing point mutations are shown in crossed boxes. Human coronary VSMCs were transiently transfected with the indicated constructs and stimulated with PPARγ ligands (10 μmol/L) as indicated for 24 hours. After stimulation, luciferase activity was assayed. Transfection efficiency was adjusted by normalizing firefly luciferase activities to renilla luciferase activities generated by cotransfection with 10 ng pRL-CMV. All experiments were repeated at least 3 times. Data are expressed as fold induction over vehicle and presented as mean±SEM (P<0.05 vs IL8-OCx6).

Figure 7. Mutations of Oct-1 motifs abrogate the activation of the GADD45 promoter after stimulation with PPARγ ligands.
EMSA experiments demonstrated that activation of PPARγ induces Oct-1 binding to its consensus sequence. EMSA from nuclear extracts isolated from both control and PPARγ ligand-stimulated HCSMCs were performed using a double-stranded Oct-1 oligonucleotide probe (Figure 8B). Specific protein-DNA complexes were undetectable in vehicle-treated cells, but increased after stimulation with 30 μmol/L RSG, TRO, and nTZDpa for 48 hours. The complex was displaced by a 100-fold molar excess of unlabeled Oct-1 oligonucleotides and supershifted in the presence of an anti-Oct-1 antibody. In contrast, excess noncompetitive double-stranded SP-1 oligonucleotides could not displace the complex. These results indicate that PPARγ ligands induce Oct-1 protein expression and increase its DNA binding-activity in HCSMCs.

To further investigate whether the induction of Oct-1 protein and DNA binding by PPARγ ligands is mediated through a receptor-dependent mechanism, we performed Western blot and EMSA analysis from nuclear extracts of HCSMCs infected with Adx-CA-PPARγ overexpressing constitutively active PPARγ. As depicted in Figures 9A and 9B, adenoviral-mediated overexpression of constitutively active PPARγ resulted in a dose-dependent induction of Oct-1 protein and DNA-binding. The mechanism by which consti-

PPARγ Ligands Induce Oct-1 Protein Expression and Binding to the Oct-1 Consensus Sequence

To determine whether activation of PPARγ directly induces Oct-1 expression in HCSMCs, cells were stimulated with RSG, TRO, and nTZDpa (30 μmol/L, 48 hours), and nuclear extracts were analyzed for Oct-1 protein expression. Oct-1 protein levels were found to be induced after stimulation with all three PPARγ ligands used (Figure 8A). PPARγ ligands, however, did not induce Oct-1 mRNA expression, indicating that the induction of Oct-1 expression involves a posttranscriptional mechanism (data not shown).
tutively active PPARγ induces Oct-1 expression occurs at a posttranscriptional level, because no induction of Oct-1 mRNA expression was observed after infection with Adx-CA-PPARγ (data not shown). Infection of HCSMCs with Adx-GFP or Adx-VP-16 exhibited no effect on Oct-1 protein expression and DNA binding. Taken together, these results suggest that the induction of Oct-1 protein expression and DNA binding by PPARγ ligands occurs through a receptor-dependent, posttranscriptional mechanism.

**Discussion**

The present study demonstrates that ligand-dependent or constitutive activation of PPARγ induces caspase-mediated apoptosis of HCSMCs. The induction of apoptosis correlates with enhanced expression of GADD45 mRNA and increased transcriptional activation of GADD45. Pharmacological inhibition of PPARγ by an irreversible antagonist prevents induction of the GADD45 promoter by PPARγ ligands, indicating a receptor-dependent mechanism. By using 5′-deletion analysis, the PPARγ-regulated elements in the GADD45 promoter have been mapped between −234 and −81, which contains one Oct-1 and one CCAAT motif. Mutation of the Oct-1 motifs in a luciferase reporter construct driven by hexamers of Oct-1 and CCAAT motifs abrogates the activation of this construct by PPARγ ligands. Furthermore, activation of PPARγ induces Oct-1 protein expression and binding to its consensus sequence, suggesting that PPARγ-mediated transactivation of the GADD45 promoter is mediated, at least in part, through increased Oct-1 expression and activity.

Pharmacological activation of PPARγ has been shown to inhibit cell growth and to induce apoptosis in VSMCs. The molecular mechanisms, however, underlying the antiproliferative and proapoptotic effects of PPARγ ligands on VSMCs are incompletely understood. Identification of downstream target genes directly regulated by PPARγ-activation relevant to growth inhibition and apoptosis might provide insights into novel vascular actions of PPARγ. We have previously reported that TZDs inhibit VSMC proliferation at the G1→S phase transition of the cell cycle by suppressing mitogen-induced phosphorylation of the retinoblastoma protein, E2F release, and expression of minichromosome maintenance proteins. In this study, we provide further evidence for an important role of PPARγ in regulating VSMC growth arrest and apoptosis. Among putative PPARγ-activated target genes associated with apoptosis and growth inhibition, we observed an induction of GADD45 expression by several PPARγ ligands.

GADD45 belongs to a subgroup of genes that are coordinately induced in growth-arrested cells. Substantial evidence implicates an important role for GADD45 in regulating cell growth. Using microinjection of a GADD45 expression vector into human fibroblasts, Wang et al. demonstrated that GADD45 induces G1 cell cycle arrest. GADD45 interacts with several cell cycle–regulating proteins, and the involvement of GADD45 in cell cycle arrest at G1/G0 or G2/M phase has been observed in different cell types. Laminar shear stress on endothelial cells leads to sustained p53 activation, a concomitant upregulation of both GADD45 and the cyclin-dependent kinase inhibitor p21^CIP1 and subsequent suppression of cyclin-dependent kinase activity causing hypophosphorylation of Rb that results in G1 or G2 arrest. In addition, Zhan et al. have shown that GADD45 inhibits the kinase activity of Cdc2/cyclinB1 and thus may induce a G2/M arrest. These observations suggest that GADD45 plays a critical role in regulating cell growth and may be an important target in PPARγ-mediated inhibition of VSMC cell cycle.

Whether GADD45 directly induces apoptosis remains controversial. Several studies have identified GADD45 as a critical mediator of apoptosis triggered by activation of the JNK and/or p38 MAPK signaling pathways. Takekawa and Saito have shown that overexpression of GADD45 induces apoptosis in HeLa cells. They observed that GADD45 binds to MEKK4, an upstream protein kinase in the signaling cascade that ultimately activates both JNK and p38 MAPK to induce apoptotic cell death. Interestingly, PPARγ ligands have been reported to activate both JNK and p38 MAPK in astrocytes and preadipocytes, although the effect was attributed to be independent of PPARγ activation. Our data support an alternative mechanism by which transcriptional upregulation of GADD45 by PPARγ promotes apoptosis in VSMCs, possibly through GADD45-dependent potentialization of JNK and p38 MAPK pathways.

TZD-induced inhibition of cellular proliferation was recently observed in mouse stem cells lacking PPARγ expression, indicating that antiproliferative effects of TZDs may occur through a receptor-independent pathway. In contrast, Bishop-Bailey et al. recently reported that induction of VSMC apoptosis by RSG at suprapharmacological concentrations is mediated through a receptor-dependent mechanism. Although GADD45 has been demonstrated to be induced by TRO in VSMCs and 15-deoxy-Δ12-prostaglandin J2 in HeLa cells, the mechanism by which PPARγ ligands upregulate GADD45, and whether it involves a receptor-dependent pathway, remains to be elucidated. To address the important issue of PPARγ-dependent versus -independent mechanisms of action, we used two different experimental approaches. In our first approach, we observed that adenovirus-mediated overexpression of a constitutively active PPARγ induced caspase-mediated apoptosis and GADD45 mRNA expression, similar to tested PPARγ ligands. In an alternate strategy, we demonstrated that PPARγ ligand-mediated induction of transcriptional activation of the GADD45 promoter was abrogated by pretreatment with an irreversible pharmacological PPARγ antagonist used to block endogenous PPARγ function. Our observations that the induction of GADD45 expression by PPARγ ligands is mediated through a receptor-dependent pathway is in accord with studies from Satoh et al. showing that an induction of GADD153, another member of the GADD-gene family, by TZD and non-TZD PPARγ ligands in HCSMCs involves a receptor-dependent pathway.

To elucidate the molecular mechanism by which PPARγ controls GADD45 expression, we first examined whether the
induction of GADD45 expression occurred principally at a transcriptional or posttranscriptional level. Observations from several studies have suggested that GADD45 mRNA expression, depending on the cell type and specific mechanism of induction, may be regulated either by increased GADD45 promoter activity or enhanced mRNA stabilization. Retinoids, which are ligands for the retinoid X receptor that heterodimerizes with PPARs to regulate target genes, have been shown to induce GADD45 mRNA through stabilization of mRNA in breast carcinoma cells. GADD45 mRNA half-life, however, did not change in HCSMCs after stimulation with TRO, indicating that PPARγ ligands had no significant effect on mRNA stabilization. In contrast, PPARγ ligands did elicit a prominent induction of GADD45 promoter activity. PPARγ can directly activate transcription of target genes by binding to PPREs and undergoing a ligand-induced conformational change which enhances interaction between the ligand binding domain (LBD) of the nuclear receptor and LXXLL motifs in transcriptional coactivators. The lack of a consensus PPRE in the human GADD45 promoter construct used in this study, however, suggested that PPARγ induces GADD45 transcription independent of direct binding to cis-regulatory elements. Using 5′-deletion constructs of the human GADD45 promoter, the PPARγ-regulated element was mapped between −234 and −81 relative to the transcription start site, a region harboring both an Oct-1 and CCAAT motif. Oct-1 belongs to the POU homeodomain family of transcription factors and is ubiquitously expressed.

Several lines of evidence indicate that the transcription factor Oct-1 may be directly involved in the regulation of the GADD45 promoter in HCSMCs. In colorectal carcinoma cells, JNK1 and ERK mitogen-activated protein kinases activate the GADD45 promoter through the Oct-1 element. The Oct-1 DNA-binding activity after DNA damage is regulated similarly to those of Zhao et al who found that induction of Oct-1 DNA-binding activity after DNA damage is regulated at the posttranscriptional level, whereas upregulation of GADD45 resulted from increased gene transcription.

In summary, data presented in this study demonstrate that ligand-dependent or constitutive activation of PPARγ promotes caspase-mediated apoptosis in HCSMCs. The ability of PPARγ activation to induce apoptosis was coupled to an induction of GADD45 expression, illustrating a novel mechanism by which PPARγ can induce growth arrest and apoptosis of VSMCs. The molecular mechanism by which PPARγ promotes transcriptional activation of GADD45 is dependent on Oct-1 function. These findings provide further support for the important role of PPARγ in regulating VSMC cell growth and apoptosis.

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References

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